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Surgical Injury and Metabolic Stress Enhance the Virulence of the Human Opportunistic Pathogen *Pseudomonas aeruginosa*

LICHENG R. WU,[1] OLGA ZABORINA,[1] ALEX ZABORIN,[1] EUGENE B. CHANG,[2] MARK MUSCH,[2] CHRISTOPHER HOLBROOK,[1] JERROLD R. TURNER,[3] and JOHN C. ALVERDY[1]

ABSTRACT

***Background*: We have shown previously that the PA-I lectin of *Pseudomonas aeruginosa* plays a key role in gut-derived sepsis during surgical stress. The aims of this study were to determine if the intestinal tract lumen of a stressed host contained soluble factors that could induce the expression of PA-I.**

***Methods*: Mice were subjected to either 30% surgical hepatectomy or sham-laparotomy, and *P. aeruginosa* was introduced into the cecum. Twenty-four hours later, feces were recovered, and PA-I and exotoxin A were determined by real-time polymerase chain reaction (PCR). In reiterative experiments, fecal filtrates from both hepatectomy and sham-operated mice were tested for their ability to induce PA-I expression in cultures of *P. aeruginosa*. Finally, the media from cultured human intestinal epithelial (Caco-2) cells stressed with excess glutamine was tested for its ability to induce the expression of PA-I in cultures of *P. aeruginosa*.**

***Results*: Both PA-I and exotoxin A mRNA were increased in vivo in the intestinal tract of mice subjected to 30% hepatectomy. Soluble fecal filtrates from hepatectomy mice induced PA-I in vitro. Media from epithelial cells exposed to excess glutamine alone induced PA-I expression.**

***Conclusions*: The intestinal environment of a stressed host contains soluble factors capable of inducing lethal virulence traits in human opportunistic pathogen *P. aeruginosa*.**

In order for bacteria to cause infection on and within an epithelial cell, intimate and dense attachment to the cell surface is necessary [1,2]. Following stress, it is generally presumed that the attachment and invasion of a given pathogen on and within the intestinal epithelium is most often promoted permissively by critical alterations in the innate and adaptive local immune response [3]. Along this line of thinking, bacterial opportunism is presumed to proceed merely as a function of a critical diminution in local mucosal defense. Recent studies, however, demonstrate that in order for mucosal pathogens to succeed in colonization and invasion of the intestinal epithelial barrier, they must actively disarm the epithelium from signaling mechanisms that recruit the migration of additional layers of immune cells [4,5].

Departments of [1]Surgery, [2]Medicine, and [3]Pathology, University of Chicago, Chicago, Illinois.

As such, several species of bacteria have been demonstrated to possess discrete immuno-evasive strategies against signals that call attention to their presence [6,7]. The ability of a particular pathogen to act in such a subversive and deceitful manner ensures its survival and successful colonization during periods of local environmental adversity and scarce nutrient resources. The recent discovery that various human pathogens display cooperative behavior and multicellular development through intricate social networks reinforces the idea that bacteria act with sophisticated "sense and respond circuitry" using soluble and contact-dependent intercellular signals [8,9]. Therefore, factors that motivate and initiate a given pathogen to invade an epithelial surface may involve a complex interplay of signals from pathogen to pathogen, host to pathogen, and finally pathogen to host. Although a substantial body of work has been produced that seeks to understand how pathogens signal the host immune response during microbial invasion, there is little information on the host–pathogen interchange in the opposite direction, namely whether factors released by the host can affect bacterial virulence expression.

Our laboratory has been interested in the mechanism by which the human opportunistic pathogen *Pseudomonas aeruginosa* transforms its phenotype from indolent colonizer to lethal pathogen within the intestinal tract of a stressed host. We have modeled such events and shown previously that *P. aeruginosa* introduced into the cecum of mice undergoing 30% surgical hepatectomy results in a near 100% mortality rate, whereas a similar inoculum has no effect when introduced into the cecum of mice following sham laparotomy [10]. We identified a virulence factor in *P. aeruginosa*, the PA-I lectin/adhesin, that appears to play a key role in attaching to and disrupting the intestinal epithelial barrier in this model. The mechanism by which *P. aeruginosa* induces lethality in the mouse following hepatectomy appears to be via a PA-I lectin–induced permeability defect to its potent and lethal cytotoxin, exotoxin A [11].

A general working hypothesis of our laboratory has been that the intestinal tract reservoir of a stressed host represents a unique niche in which microbial organisms are "cued" to change their phenotypes to those of more virulent and lethal strains. The notion that bacteria possess sensory input systems to sense their population density and environment has been fueled by the discovery of auto-inducer or pheromonal molecules that these organisms use to orchestrate complex assemblage behavior [12]. This intricate bacterial cell-to-cell communication pathway arms *P. aeruginosa* with a sense-and-respond circuitry capable of integrating a variety of external stimuli such as bacterial population density, nutrient composition, and physico-chemical changes (e.g., hypoxia, pH) in the local microenvironment. One such system, the quorum sensing signaling system, uses homoserine lactone (HSL) molecules for intercommunication networking which, upon proper cue, can activate hundreds of genes involved in phenotypic virulence expression. Our laboratory and others have shown that the secreted and freely diffusible quorum sensing signaling molecule C4–HSL is a strong inducer of the PA-I lectin/adhesin in *P. aeruginosa* [13,14]. Given the ability of *P. aeruginosa* to utilize the quorum sensing signaling system ultimately to up-regulate its virulence, in the present study we hypothesized that the PA-I lectin/adhesin of *P. aeruginosa* might be "in vivo expressed" by factors unique to the intestinal tract of a stressed host, thus accounting for its enhanced lethality in this model. Therefore, the specific aims of this study were to determine if the intestinal tract of a stressed host contained soluble factors that could alone induce the expression of the PA-I lectin/adhesin in *P. aeruginosa*. In an effort to isolate the specific factors that might elicit such a response, we examined the ability of Caco-2 cultured human epithelial cells, subjected to a metabolic shift induced by excess glutamine, to induce PA-I expression.

MATERIALS AND METHODS

Experimental protocol

Mouse experiments. All experiments were approved by the Animal Care and Use Committee at the University of Chicago. In-bred Balb/c

mice weighing 20–25 g were used for all experiments. Mice were kept in individual wire-bottom cages to avoid coprophagia during the entire experimental period. In order to determine if the intestinal environment of a stressed host induces *P. aeruginosa* to express enhanced virulence, strains of *P. aeruginosa* (PA27853), (American Type Culture Collection, Manassas, VA) were injected directly into the cecum of mice undergoing either sham laparotomy or 30% hepatectomy (n = 6 per group). At 24 h later, cecal contents were collected and total RNA was extracted to determine mRNA levels of PA-I and exotoxin A by real-time reverse transcriptase–polymerase chain reaction (RT-PCR). Next, in order to determine if soluble factors from the intestinal tract of a stressed host could induce virulence expression in *P. aeruginosa*, fecal contents were obtained from mice subjected to sham laparotomy or 30% surgical hepatectomy (n = 6 per group). Cell-free supernatants of fecal samples from the cecum of mice were obtained by first excising a fixed area of cecum, cutting it open, and washing it in 2 mL tryptic soy broth. Next, the cecal tissue was removed and the remaining fecal suspension dissolved and separated by spinning down the sample at 5000 g for 5 min. Finally, the supernatant was filtered through a 0.22-micron filter (Millipore, Billerica, MA) and immediately added to freshly growing bacterial fusion reporter constructs of *P. aeruginosa* (PA27853/PL-EGFP) at 1:20 dilution. Six runs of each experiment were performed in triplicate per group. Wild-type PA27853 were also exposed to fecal filtrates and analyzed for PA-I by Northern blot analysis.

Cultured epithelial cell (Caco-2) experiments. In order to determine whether soluble products released into the intestinal lumen following surgical stress that induces PA-I expression in *P. aeruginosa* were of epithelial cell origin, we exposed strains of PA27853 to cell culture media from Caco-2 cells enriched with increased concentrations of glutamine, an amino acid whose uptake, utilization, and metabolism by epithelial cells is highly affected by stress [15,16]. An additional reason to study the effect of media from epithelial cells exposed to glutamine is that glutamine changes the epithelial cell redox state and the redox state of the surrounding media by affecting intracellular glutathione concentrations [17]. Changes in extracellular redox potential are a well-established mechanism by which bacteria induce gene expression via redox-sensitive transcriptional regulators [18]. In addition, in our mouse model of hepatectomy, the redox state in the cecum is markedly altered compared to sham-operated mice [10]. Therefore we harvested media from Caco-2 cells grown to confluence, enriched with L-glutamine 300 mcg/mL/day for 3 days, and compared this media to Caco-2 cells without enriched L-glutamine at day 1 and 2 of culture. In addition, we ran parallel studies using glutamate and glycine to determine the specific effect of glutamine on this response. We measured the redox state of the media in all groups. Freshly harvested media was filtered through a 0.22 micron filter and added immediately to freshly growing PA27853. Messenger RNA for PA-I was determined by Northern blot in PA27853. Reiterative experiments were performed with glutamate and glycine for comparison. The PA-I fusion reporter constructs of *P. aeruginosa* (PA27853/PL-EGFP) were also exposed to the Caco-2 cell culture media enriched with L-glutamine (300 mcg/mL) every day for 3 days, and PA-I expression was determined in cytosolic fractions of PA27853/PL-EGFP by fluorometry.

Bacterial strains, epithelial cell lines

Pseudomonas aeruginosa strain ATCC 27853 (PA27853) was used in all experiments and is a non-mucoid clinical strain isolated originally from a blood culture. Caco-2 cells are well-characterized human colon epithelial cells that maintain a stable transepithelial electrical resistance (TEER) when grown confluently in culture [19].

Construction of the GFP PA-I reporter strain PA27853/PL-EGFP

In order to develop a reporter strain of *P. aeruginosa* that would respond to elements of host stress, we created a fusion construct that included key promoter elements of both the quorum sensing signaling system and the RpoS system. Expression of the PA-I lectin, as well as hundreds of other virulence genes in *P. aerugi-*

nosa, has been shown to be dependent on both the quorum sensing signaling system and RpoS. Therefore, in the construction of our reporter strain, two key elements, the lux box element of the quorum sensing signaling system and a sigma factor RpoS consensus were located within 112 bp from the starting ATG site [13]. Next, to create PA-I reporter strain pUCP24/PL-EGFP, a region of 470 bp, including a 440-bp upstream plus a 30-bp of N-terminus of *PA-1* except stop codon, was amplified from PA27853 DNA, and restriction sites *KpnI* and *XbaI* were inserted using respective primers: F: 5′-GGTACCCCGGTTCGACCCCG-GCTCCGG-3′, R: 5′-TCTAGAGATTGATCTC-CGATATATGAAT-3′. *Egfp* was fused with *pl* via *XbaI* restriction site, and pUCP24/PL-EGFP were electroporated into PA27853 electrocompetent cells. *Pseudomonas aeruginosa* constructs were selected on the basis of gentamicin (100 mcg/mL) resistance and fluorescence induction by C4-HSL (0.1 mM) for PA27853/PL-EGFP.

Mouse model

Animals were anesthetized (ketamine 100 mg/kg, xylazine 10 mg/kg, atropine 0.04 mg/kg) intraperitoneally. Through a midline incision, the floppy left lobe of liver was cauterized using electrocautery, which resulted in minimal bleeding. Specimens were weighed, and the ratio of excised liver to body weight (1.2–1.4%) was calculated to confirm uniformity of the resection. A puncture into cecum with a 27-gauge needle was used to inject bacteria. One hundred microliters of *P. aeruginosa* 27853 were grown in the tryptic soy broth overnight (about 10^9 cfu/mL), then 200 microliters of diluted *P. aeruginosa* (about 10^7 cfu/mL) was injected. The cecal puncture site was ligated with a 4-0 silk suture and swabbed with Acudyne (Rohm and Haas, Philadelphia, PA), washed with sterile saline, and the abdomen closed. We have had no episodes of peritonitis or spillage. Animals were allowed only water ad libitum for the remainder of the study period. This model results in 100% mortality within 48 h after the procedure. Animals died a typical septic death with features of chromodacryorrhea, lethargy, scant diarrhea, and ruffled fur. Animals were sacrificed when they were moribund. Sham-operated control animals injected with identical doses of *P. aeruginosa* appeared well.

Quantitation of PA-I and exotoxin A using real-time PCR from fresh fecal extracts

At 24 h following introduction of PA27853 into the cecum of sham-operated or hepatectomized mice, feces were retrieved from the cecum and processed immediately in phosphate buffered saline (PBS) to separate the fecal matter into a crude fraction. After sedimentation for 1 min, the supernatant was spun down and washed with PBS. The bacterial fraction was mixed with two volumes of RNA Protect Bacteria Reagent (Qiagen, Germany) for 5 min. Lysozyme (250 mcg/mL) was used to digest the bacterial fraction for 5 min at room temperature, and RNA was immediately extracted by TRIzol LS reagent (Invitrogen, Carlsbad, CA). Phase-lock gel was used to separate the aqueous phase from organic phase (Eppendorf, Germany). DNase treatment was performed in a column using RNase kit (Qiagen, Germany). The RNA was quantitated by both spectrophotometer and with formaldehyde agarose gel electrophoresis. Two micrograms of RNA was reverse transcripted using Super Script™ II RNase H–reverse transcriptase and random primers (Invitrogen, Carlsbad, CA). Taqman primers and probes (Table 1) were designed on the basis of annotated *P. aeruginosa* PAOI genome, using Primer Express 1.0 software (Perkin-Elmer Applied Biosystems, Foster, CA). Probes were labeled with the reported dye fluorescein (Fl) at the 5′ end and with Black Hole Quencher+5′ Fluorescein (BHQ~Fl) at the 3′ end. Quantification of cDNA was performed on the Thermal Cycler GeneAmp 7700 Sequence detection system (Perkins-Elmer Applied Biosystems, Foster, CA). For each gene, the amount of cDNA was calculated based on Ct levels and normalized to the housekeeping gene, citrate synthetase.

Assessment of bacterial fluorescence measured by fluorometry of cytosolic fractions

27853P/PL-EGFP cultured overnight in TSB-Gm (5 mcg/mL) was diluted at 1:20 in TSB-Gm free and incubated for 4 h, followed by the treatment with different stimulators (fecal fil-

trate from mice; Caco-2 cell culture medium). After 4 h of treatment, PL-EGFP/27853 strains were washed with PBS and re-suspended in sonication buffer (50 mM NaH_2PO_4, 10 mM TrisHCl, pH 8.0, 200 mM NaCl). The bacteria suspension was disrupted using a Sonic Dismembrator (Fisher, St. Louis, MO). After removal of debris by centrifugation, the fluorescence of the cytosolic fraction was measured with a Versafluor™ Flurometer (Bio-Rad, Hercules, CA). Filters 480/20 and 510/10 were used for excitation and emission, respectively. Total protein concentration in the fractions was measured using Micro-BCA method (Pierce Biotechnology, Rockford, IL). The relative changes of fluorescence were based on the fluorescence intensity normalized to total protein concentration.

Assessment of bacterial fluorescence by fluorescent microscopy

The effect of the various fecal filtrates on the activation of fluorescence in the PA-I reporter strain PL-EGFP/27853 was assessed under static conditions using Axiovert TM 100 fluorescent microscopy (Carl Zeiss, Thornwood, NY). One hundred microliters of the bacterial culture was grown to log phase and mixed with 1 mL of Ringer's solution with/without feces filtrate (1:20 dilution) and poured into delta dishes (Bioptech, Butler, PA). The fluorescence intensity of bacteria was observed directly by fluorescence microscopy using DIC and GFP filters. Interval lapsed exposures were used to follow the changes in fluorescence as a time-dependent function for 3 h. Temperature was adjusted with a Bioptechs thermostat temperature control system. Tungsten lamps (100 W) were used for both DIC and GFP excitation.

PA-I mRNA quantization using Northern blot

Total RNA of *P. aeruginosa* was isolated by the three-detergent method [20]. The RNA pellet was re-suspended in RNase-free water and quantitated spectrophotometrically. The RNA (10 mcg) was separated by gel electrophoresis and transferred to hybond nylon membranes (Amersham, Piscataway, NJ). After cross-linking, membranes were prehybridized in xotch solution. A specific cDNA probe for both PA-I and 16S was radiolabeled with α^{32}P-dCTP using a random primer labeling kit (DECA primer II, Ambion, Austin, TX). The probe was generated by PCR using PA-I and 16S primer, and cloned into PCR2.1 vector (Invitrogen) and sequenced, which matched PA-I and 16S, respectively. PA-I primers were F(accctggacattattgggtg) R(cgatgtcattaccatcgtcg); 16S primers were F(ggacgggtgagtaatgccta) R(cgtaagggccatgatgactt). Membranes were then incubated with labeled probes for 18 h. Membranes were washed twice in 2 × SSC/0.1% SDS for 15 min and then three times in 0.2 × SSC/0.1% SDS at 60°C. The membranes were restriped using restrip buffer and rehybridization.

Redox status of Caco-2 cell culture medium measured by ORP

The redox status of each solution was measured using an oxidation/reduction probe (ORP, Thermo Orion, Beverly, MA). The platinum redox electrode was immersed into the sample until readings were equilibrated fully. The redox potential of the sample solution was expressed as total electric potential in the millivolt mode. The probe was zeroed repeatedly with Orion ORP standard (967961) before each measurement.

RESULTS

Virulence proteins associated with lethal phenotypes for intestinal P. aeruginosa, *the PA-I lectin/adhesin and exotoxin A, are expressed in vivo in the mouse cecum following surgical hepatectomy and short-term starvation*

Twenty-four h following the direct introduction of *P. aeruginosa* (PA27853) into the cecum of mice, the abundance of PA-I and exotoxin A mRNA measured by real-time PCR was significantly increased in feces from mice subjected to hepatectomy compared to sham-operated controls ($p < 0.001$; Fig. 2). When *P. aeruginosa* fusion constructs (PL-EGFP) were exposed to acellular filtered fecal contents from the cecum of both groups of mice, fluorescence was visibly increased by filtered fecal contents from hepatectomy mice versus sham-operated controls (Fig. 3I). When PL-EGFP was exposed to

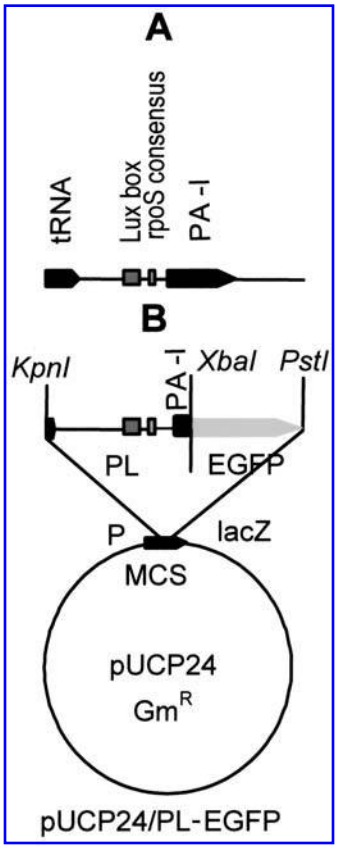

FIG. 1. PA-I reporter construction. **A.** shows the entire PA-I gene and its upstream regulator region in strain PA27853. The *lux* box type element of quarum sensing signaling system and RpoS(Δ^S) consensus are shown. **B.** Fusion construct. *PL* region 440 bp upstream + 30 bp of N-terminus of *pa-I* minus stop codon) was fused with *egfp* amplified from pBI-EGFP and introduced into pUCP24 *E. coli-P. aeruginosa* shuttle vector, which was electroporated into PA 27853 to create PA27853/PL-EGFP.

fecal filtrates from mice subjected to hepatectomy, both clumping and biofilm production was seen. Fecal filtrates from mice subjected to a 30% hepatectomy induced a statistically significant increase in PL-EGFP fluorescence of cytosolic fractions of bacteria (Fig. 3II). Northern blot confirmed that fecal filtrates from mice subjected to 30% hepatectomy increased PA-I mRNA levels of *P. aeruginosa* (Fig. 3III).

Media from human cultured intestinal epithelial cells (Caco-2) exposed to increased concentrations of glutamine results in a change in redox state and induces PA-I expression in P. aeruginosa

Media from Caco-2 cells exposed to a daily supplement of L-glutamine for 3 days increased the abundance of PA-I mRNA compared to non-supplemented media or media supplemented with L-glutamine on day 3 only (Fig. 4I). A similar effect of glutamine supplementation was also observed in fluorescence of cytosolic fractions in the reporter construct PL-EGFP (Fig. 4II). The redox state of the media was altered by the individual treatments and appeared to demonstrate the greatest change (more oxidative) in media from cells supplemented with excess L-glutamine daily for 3 days (Table 2). Reiterative experiments with glutamate and glycine showed no such changes in redox state and did not induce the expression of PA-I mRNA (data not shown). Taken together, these data suggest that intestinal epithelial cells exposed to an increased amount of glutamine, as might occur during surgical stress, may alter the extracellular redox state resulting in signaling of *P. aeruginosa* virulence. The clinical relevance of this observation is applicable to surgical stress where the extracellular redox state is significantly altered [10].

DISCUSSION

This study builds on our previous work and confirms that the state of virulence of the hu-

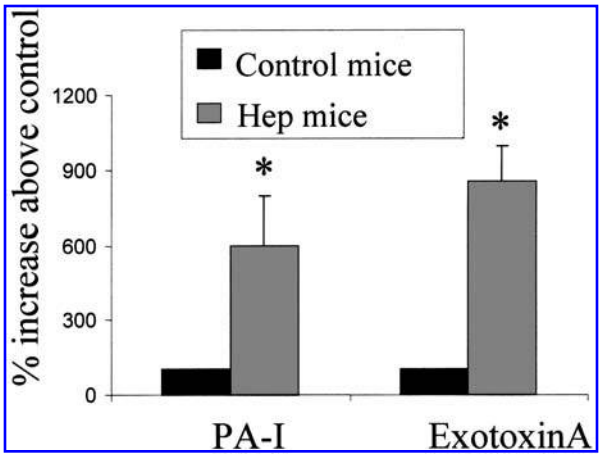

FIG. 2. PA-I and exotoxinA mRNA of *P. aeruginosa* 27853. The levels of both PA-I and exotoxin A mRNA were increased (*$p < 0.001$, one-way ANOVA) in fecal samples harvested 24 h following direct injection of *P. aeruginosa* into the cecum of mice subjected to 30% hepatectomy (Hep). mRNA levels were normalized to the housekeeping gene, citrate synthetase to control for mRNA extraction.

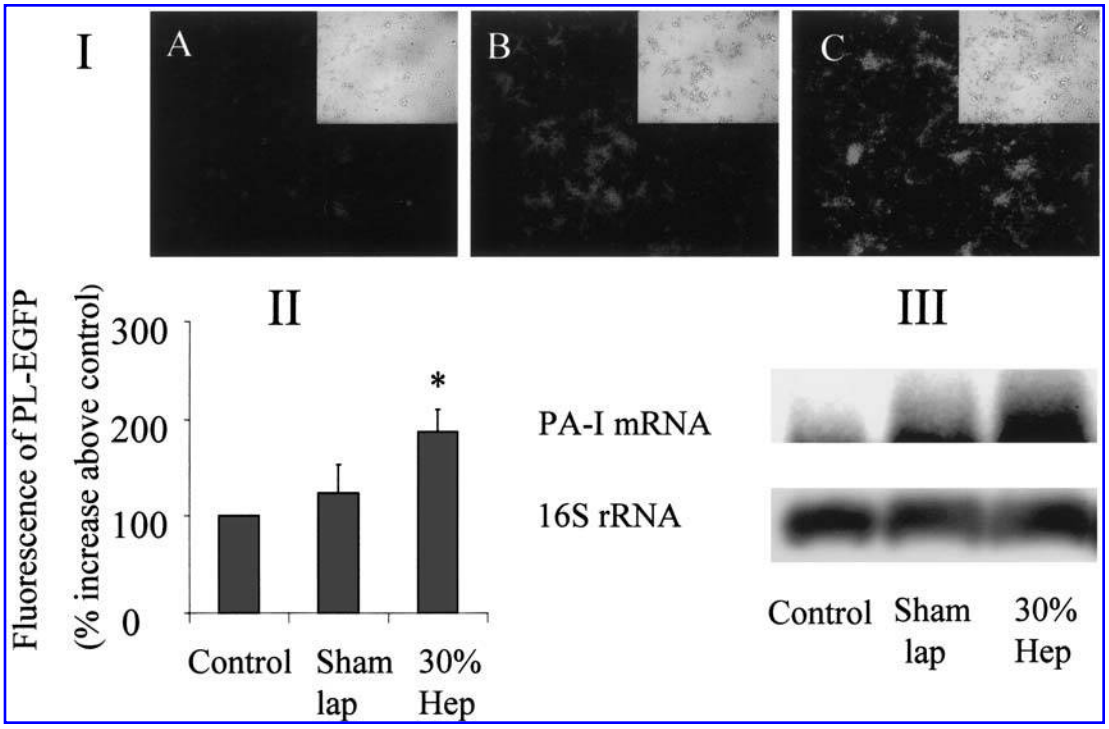

FIG. 3. **I.** Microscopic overlays of fluorescence of PL-EGFP/27853 exposed to tryptic soy broth only (**A**) or to processed fecal filtrates obtained from mice subjected to sham operation (**B**) or 30% surgical hepatectomy (**C**). Overlays in the corner are images demonstrating the density of the bacterial sample. Images demonstrate that fecal filtrates from 30% hepatectomy mice induce an increase in PL-EGFP/27853 fluorescence compared to either sham operated or control fecal filtrates. Fecal filtrates from both sham-operated controls and 30% hepatectomy mice induced clumping in bacteria, a precursor to biofilm formation. **II.** Quantitative assessment of fluorescence of PL-EGFP/27853 exposed to fecal filtrates harvested from sham-operated control and 30% hepatectomy. A significant increase in the fluorescence of cytosolic fractions of PL-EGFP/27853 (*$p < 0.05$, unpaired t-test) exposed to fecal filtrates was observed in mice subjected to 30% surgical hepatectomy. **III.** Northern blot analysis confirmed the findings in reporter strains.

man opportunistic pathogen *P. aeruginosa* can be enhanced by soluble factors within the intestinal tract of a stressed host. Several novel observations have been described herein which demonstrate clearly that soluble factors within the intestinal lumen, and released by epithelial cells themselves, are capable of inducing a critical virulence gene in *P. aeruginosa*, the PA-I lectin/adhesin (*lecA*). We used the PA-I lectin/adhesin of *P. aeruginosa* as the virulence gene of interest as we have shown previously that this virulence-related attachment factor plays a key role in lethal gut-derived sepsis in mice following surgical stress (hepatectomy). In selected experiments, we also measured exotoxin A as its permeation across the intestinal epithelium into the systemic compartment, facilitated by a PA-I–induced permeability defect, appears to mediate the lethal toxemia of intestinal *P. aeruginosa*. Both PA-I and exotoxin A are co-regulated genes under control of the quorum sensing signaling system [21].

The main aim of this study was to determine if PA-I expression in *P. aeruginosa* could be induced by soluble components present in the intestinal lumen during catabolic stress. To our knowledge, the notion that stress releases soluble products into the intestinal lumen capable of inducing virulence expression in colonizing microbes has not been tested formally. We used three separate yet redundant approaches to determine whether PA-I is indeed expressed in vivo by conditions within the intestinal tract of a stressed host. The use of the fusion construct 27853/PL-EGFP has a major advantage in that several copies of the plasmid are introduced into the organism, making it a more sensitive reporter for PA-I expression. In addition, this construct was designed specifically to contain several elements of the quorum sensing sig-

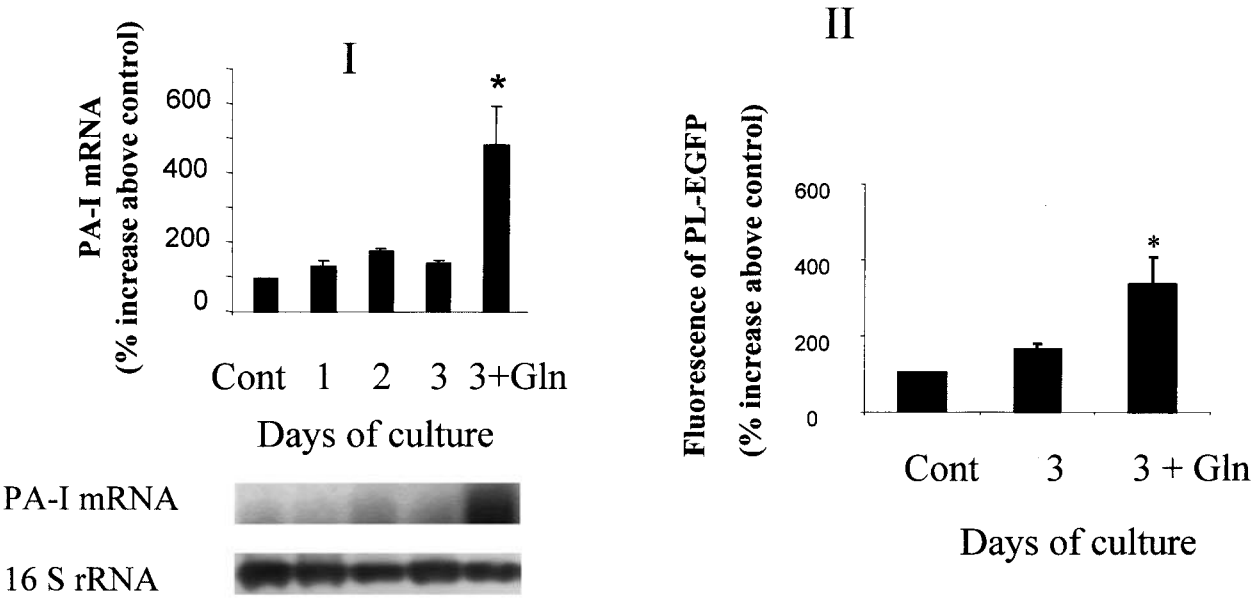

FIG. 4. **I.** Northern blots of PA-I mRNA induced by the exposure of strain 27853 to media from Caco-2 cells cultured 3 days without daily supplementation (1, 2, 3) and exposed to media in which excess glutamine (300 μg/mL) was added daily (3+Gln). Results demonstrate a statistically significant ($^{+}p < 0.01$, one-way ANOVA) increase in PA-I mRNA in 27853 when exposed to media supplemented daily for 3 days with 300 μg/mL of L-glutamine. **II.** Fluorescence of cytosolic fractions PL-EGFP/27853 exposed to media from Caco-2 cells cultured for 3 days with and without daily supplementation of excess L-glutamine. Results demonstrate a statistically significant ($^{+}p < 0.01$, one-way ANOVA) increase in the fluorescence of PL-EGFP/27853 exposed to media supplemented daily for 3 days with 300 μg/mL of L-glutamine.

naling system so as to function effectively as a highly sensitive reporter strain capable of "sensing" host stress. As a second approach, we harvested fresh feces and performed real-time PCR so as to be able to detect very small amounts of PA-I mRNA. This approach allowed us to capture a realistic "snapshot" of the in vivo virulence of injected strains within the lumen as they responded to the true environment of either sham-operation or 30% hepatectomy without the need for subculture strains, as was reported previously by our group. Finally, we used the media from cultured human intestinal epithelial cells to determine whether factors that activate PA-I expression are present within the intestinal epithelium itself and whether such factors could be expressed into the lumen under conditions of metabolic stress (excess glutamine).

Results from the present study demonstrated that surgical stress, in the form of a surgical hepatectomy and short-term starvation, created an intestinal environment that activated virulence in *P. aeruginosa*, as judged by an increase in the fluorescence of 27853/PL-EGFP. These results were complemented by experiments in

TABLE 1. PRIMERS AND PROBES USED IN REAL TIME POLYMERASE CHAIN REACTION

Gene	*Sequence*
pa-1	5′ GGTGCGCTGGTCATGAAGA 3′
	5′ ACGGAACAACCCGGTATTGA 3′
	5′ [Fl]TGGCAACAGCGGAACCATTCCG[BHQ~Fl] 3′
exA	5′ GAACGCCGGTAACCAGCTC 3′
	5′ CCTGACGAAGAAGGTGGCA 3′
	5′ [Fl]CATGTCGCCGATCTACACCATCGAGA[BHQ~Fl] 3′
cs	5′ GCCGCCATGGTGTACAAGTA 3′
	5′ GTTCAGGTCGTTACGCGGAT 3′
	5′ [Fl]TCCAAGGGCGAGCCGATGATG [BHQ~Fl] 3′

Table 2. The Redox State of the Caco-2 Cell Media Treated with L-Glutamine

	Redox state mV (Mean ± SD)
Media alone − no glutamine	−22.83 ± 1.45
Media alone + glutamine	28.68 ± 1.66
Media alone + glutamate	−13.73 ± 1.05
Media alone + glycine	−17.96 ± 2.88
Media + Caco-2 cells measured on day 1	−29.64 ± 1.02
Media + Caco-2 cells measured on day 2	−39.12 ± 1.69
Media + Caco-2 cells measured on day 3	−40.66 ± 2.14
Media + Caco-2 cells + L-glutamine measured on day 3[a]	−59.82 ± 1.31

[a]Media supplemented daily with 300 μg/mL L-glutamine

which mRNA for PA-I and exotoxin A was assessed following the introduction of live *P. aeruginosa* into the cecum of hepatectomized or sham-operated mice. Taken together, these studies strongly suggest that intestinal pathogens such as *P. aeruginosa* have the capacity to sense catabolic stress and respond accordingly.

In order to determine what role the intestinal epithelium itself played in the above response, we designed studies using human colon epithelial cells (Caco-2). In pilot experiments, we found that the media from unstressed Caco-2 cells cultured to confluence alone had no effect on *P. aeruginosa* PA-I expression. In an attempt to recapitulate the metabolic stress that the epithelium might undergo during a catabolic surgical injury, we added supplemental glutamine to the culture media. We specifically chose this approach over directly exposing Caco-2 to stress conditions, so as not to disrupt their membrane integrity. We focused on glutamine over other amino acids because a basic response of the intestinal epithelium during stress is to change its uptake and metabolism of glutamine [22]. Furthermore, Jonas et al. [23] and others have shown recently that glutamine regulates the extracellular redox state of Caco-2 cells—an effect which might trigger redox-sensitive transcriptional regulators in gram-negative bacteria leading to virulence gene expression [24,25]. We postulated that the beneficial effect of glutamine uptake by the intestinal epithelium in response to stress might paradoxically induce bacterial virulence gene expression in *P. aeruginosa*, as judged by an increase in its PA-I lectin [26,27]. Therefore, we measured the effect of the media of Caco-2 cells exposed to excess amounts of glutamine on PA-I expression in *P. aeruginosa* in association with changes in the extracellular redox state. Results of these studies suggested that PA-I expression in *P. aeruginosa* responded to media from Caco-2 cells exposed to excess glutamine, and was associated with a significant shift in the extracellular redox state measured in media. Therefore, increased transcription of PA-I lectin/adhesin may indeed be caused by a change in the redox potential or the other factors produced by stressed colonic epithelial cells. Whether the effect observed in the present study is indeed a result of the degree of redox change, as has been shown for other pathogens such as *E. coli*, will require further clarification [28,29]. More comprehensive studies are now underway to examine the relationship between nutrient composition and oxidation/reduction potential of cultured intestinal epithelial cells.

That an amino acid such as L-glutamine might benefit the intestinal epithelium while at the same time be metabolized to an end-product that signals a pathogen such as *P. aeruginosa* makes sense from the standpoint of the pathogen. In the best interest of the pathogen, it stands to reason that opportunism might be signaled by soluble products of host stress, especially those that result from metabolic stress. The findings from this study could explain why glutamine supplementation in clinical trials has not led consistently to improved outcome from sepsis [30,31]. In fact, in one recent prospective trial in which oral glutamine was administered to critically ill patients, mortality was actually increased [32]. Data from the present study also tempt some interesting speculations on the interplay between a stressed host and its colo-

nizing pathogens, especially when confronted by opportunistic pathogens such as *P. aeruginosa*. Within several days of critical illness, *P. aerguinosa* is present in the feces of more than 50% of patients [33]. The probability that *P. aeruginosa* might express a lethal phenotype within the intestinal tract of a stressed host is likely governed by local factors within the intestinal lumen that induce the organism to express a persistently adhesive and cytotoxic phenotype. Interestingly, the same system that enhances many virulence genes in *P. aeruginosa*, the quorum sensing signaling system, also regulates the production of protective biofilms, enabling *P. aeruginosa* to mount a toxic offensive from an epithelial surface at arm's length from the immune system [34]. Given the growing problem of antibiotic resistance to *P. aeruginosa*, a more precise understanding is needed of the mechanisms by which opportunistic organisms within the intestinal tract reservoir are induced to express subversive virulence traits in response to host stress.

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Address reprint requests to:
Dr. John Alverdy
Department of Surgery
Center for Surgical Infection Research
University of Chicago
Pritzker School of Medicine
5841 S. Maryland MC 6090
Chicago, IL 60637

E-mail: jalverdy@surgery.bsd.uchicago.edu

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